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REVIEW

PAK signaling in oncogenesis

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The p21-activated kinase (PAK) family of serine/threonine kinases is important in physiological processes including motility, survival, mitosis, transcription and translation. PAKs are evolutionally conserved and widely expressed in a variety of tissues and are often overexpressed in multiple cancer types. Depending on structural and functional similarities, the six members of PAK family are divided into two groups with three members in each group. Group I PAKs are activated by extracellular signals through GTPase-dependent and GTPase-independent mechanisms. In contrast, group II PAKs are constitutively active. Over the years, accumulating data from tissue culture models and human tumors has increased our understanding about the biology of PAK family members. In this review, we have summarized the complex regulation of PAK and its downstream diverse myriads of effectors, which in turn are responsible for the biological effects of PAK family of kinases in cancer cells.
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Introduction

The salient features of a cancer cell include loss of homeostasis, altered cytoskeleton dynamics, uncontrolled cell proliferation, escape from apoptotic signals and deregulated gene products. A plethora of evidence points to functions for the p21-activated kinase (PAK) family of serine/threonine kinases in each of the above processes. PAKs are either upregulated or hyperactivated in a variety of human cancers, such as breast, ovary, colorectal, thyroid and pancreatic (Kumar *et al*., 2006). In a mouse model, PAK1 hyperactivation is sufficient to form mammary gland tumors (Wang *et al*., 2003, 2006). Although it appears promising to consider PAKs as potential therapeutic targets for interrupting cancer progression, designing a PAK-specific inhibitor has been a challenge, in part owing to the functional similarities among the PAK family members. Effective targeting of PAKs will depend on our knowledge of PAK's activation and its impact on downstream signaling cascades leading to phenotypic changes relevant to tumor development and progression.

PAK was initially identified as a binding partner of the Rho GTPases Cdc42 and Rac1 (Manser *et al*., 1994). Thus far, six PAK family members have been identified in mammalian cells (Hofmann *et al*., 2004; Figure 1). On the basis of structural and functional similarities, we can categorize the PAK family into two subgroups, with three members in each. Group I includes PAK1, PAK2 and PAK3, and group II includes PAK4, PAK5 and PAK6 (Figure 2). All PAKs consist of a C-terminal kinase domain and an N-terminal regulatory domain containing a GTPase-binding domain and an inhibitory domain. However, group I and II PAKs share only about 50% of the GTPase-binding and kinase domains (Bokoch, 2003; Kumar *et al*., 2006). Group I PAKs are activated by GTPases such as Cdc42, Rac, TC10, CHP and Wrch-1, as well as in a GTPase-independent manner.

Group I PAKs

The group I PAK exists as inactive homodimers, wherein the two kinase domains from two different molecules inhibit one another (Pirruccello *et al*., 2006). The kinase inhibitory domain (KID) of PAK1 binds to the kinase domain of its counterpart and keeps it in an inactive state. GTP-bound forms of Cdc42 and Rac bind to the regulatory domain of the kinase and displace it, thereby allowing phosphorylation of the kinase domain. Conserved residues within the N-terminal p21-binding domain (PBD) participate in the binding and activation by the small GTPases. There is a short lysine-rich stretch (PAK1 residues 66–68) just upstream of the Cdc42 and Rac interactive-binding (CRIB) domain within the PBD region for effective binding of the small GTPases (Lei *et al*., 2000). In addition to the PBD domain, the regulatory domain contains two canonical PXXP Src homology 3 (SH3) binding motifs and a nonclassical SH3-binding site for PAK-interacting exchange (PIX) factor. The first canonical SH3 site has the capability to bind to the adapter protein Nck, whereas the second SH3 site can bind Grb2. Membrane recruitment of PAK1 by SH3-containing Nck and Grb2 adapter proteins results in

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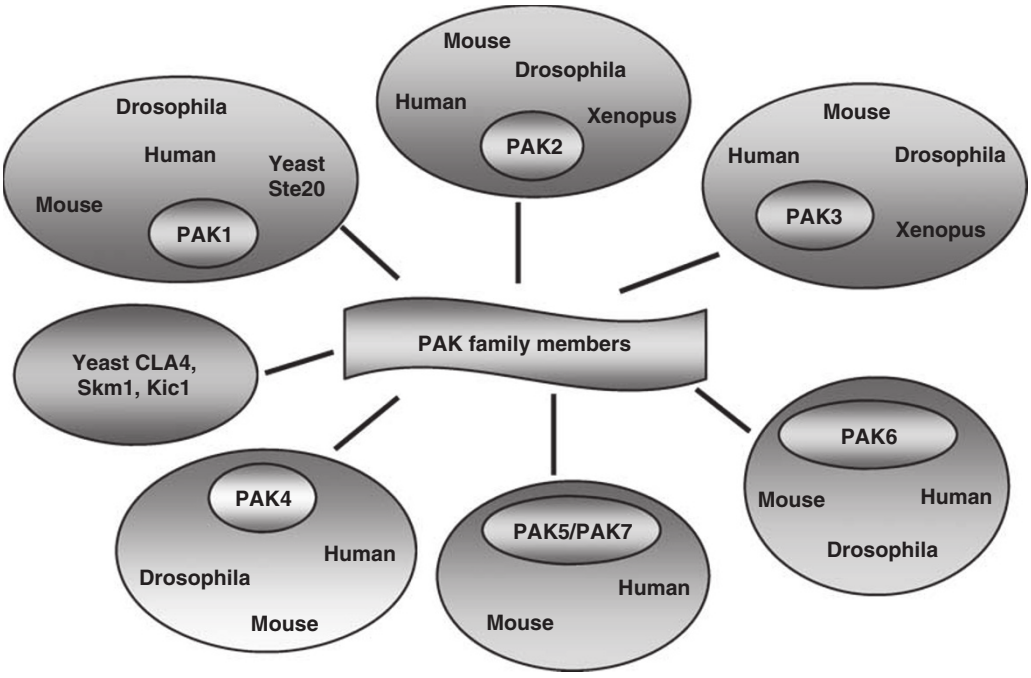

Figure 1 p21-activated kinase (PAK) family members. PAKs are evolutionarily conserved. On the basis of structural and functional similarities, the six members of human PAK family can be categorized into two subgroups—group I consisting of PAK1, PAK2 and PAK3, and group II consisting of PAK4, PAK5/PAK7 and PAK6. The PAK homologues in lower organism with functional similarities are included in each of the subgroups.

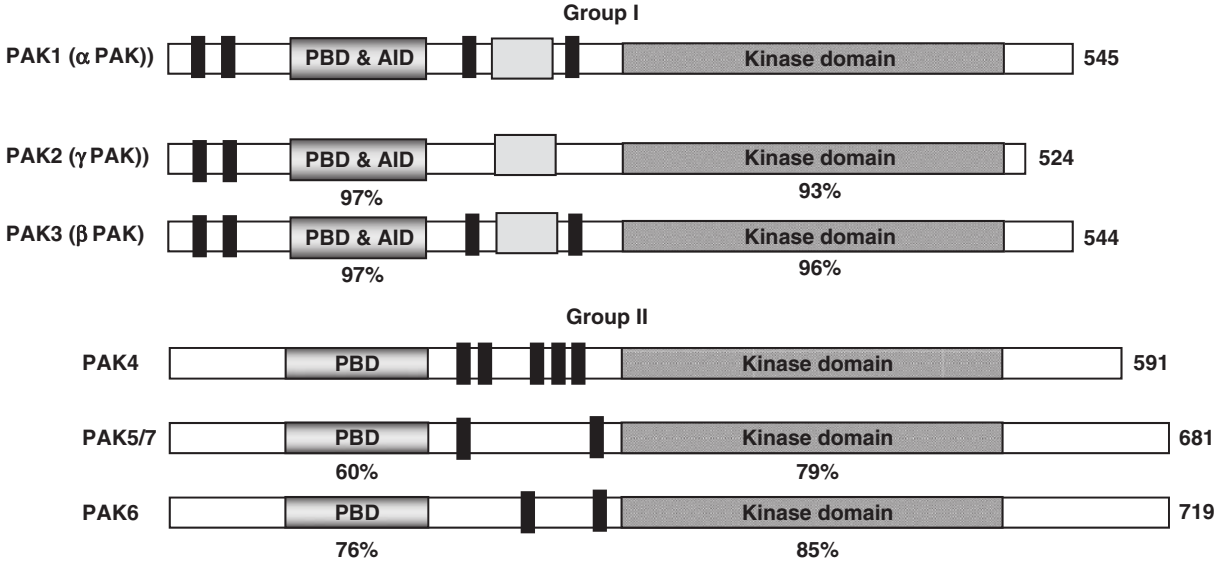

Figure 2 Structural domains of group I and group II p21-activated kinases (PAKs). The group I PAKs contain a conserved PBD/AID. Binding of GTPases Cdc42 and Rac to the PBD releases it from the kinase domain. The group II PAKs contain a PBD but lack AID. Only group I PAKs have PIX-binding region but all PAKs have conserved proline-rich motifs. Percentage similarities within the group for PBDs and kinase domains are indicated. PBD, p21-binding domain; AID, autoinhibitory domain; ▮ proline-rich region; ▯ PIX-binding domain.

the stimulation of its kinase activity through either phosphorylation by 3-phosphoinositide-dependent kinase-1 or interaction with lipids such as sphingosine or phosphatidic acid (Bokoch, 2003). PAKs can also be activated by G-protein-coupled receptor kinase-interacting target 1 (GIT1), which associates indirectly with PAK by the focal adhesion-associated protein PIX, through a mechanism that does not require the small GTPases (Bokoch, 2003; Hoefen and Berk, 2006). In addition, PAK1 can be directly activated by Akt, and PAK2 can be activated through cleavage by caspase 3 (Walter *et al*., 1998; Zhou *et al*., 2003).

To date, seven autophosphorylation residues have been mapped for PAK1: Ser-21, Ser-57, Ser-144, Ser-149, Ser-198, Ser-203 and Thr-423. Of these, autophosphorylation of Thr-423 is important for counteracting autoinhibition and maintaining a full catalytic function toward its substrates. Phosphorylation of Ser-21 and Ser-144 also contributes to kinase activation, whereas autophosphorylation of Ser-198 and Ser-203 in PAK1 serves to downregulate PIX–PAK interaction (Chong *et al*., 2001). PAK2 is autophosphorylated at eight sites: Ser-19, Ser-20, Ser-55 and Ser-192; and Ser-197, Ser-141, Ser-165 and Thr-402. Of these, the first six sites are autophosphorylated by Mg-ATP alone whereas the latter three sites—Ser-141, Ser-165 and Thr-402—are selectively phosphorylated upon PAK2 activation (Gatti *et al*., 1999; Jung and Traugh, 2005). Irrespective of the mode of activation, activated PAKs phosphorylate their substrate/effector proteins, which in turn activate various biological functions (Figure 3).

Cytoskeleton remodeling a prerequisite for invasion

The high mortality rate associated with cancer is due to tumor metastasis, which involves the invasion of primary tumor cells through tissue and the extracellular matrix (ECM) to distant sites, a process that requires cytoskeleton remodeling. A complex interplay of signaling cascades triggers changes in the cytoskeletal dynamics, leading to a motile phenotype, and PAKs are considered prime regulators of the actin cytoskeleton and motility (Sells *et al*., 1997). However, depending upon the precise protein–protein interactions and the regulation of PAK at the plasma membrane, PAK has different effects on the actin cytoskeleton and cell motility. Overexpression of activated PAK mutants induces loss of stress fibers and focal adhesion complexes, whereas modest levels of PAK mutant expression induce polarized lamellipodia and increased cell motility (Manser *et al*., 1997; Sells *et al*., 1997). The basis for these differences in phenotype is not completely understood but involves the phosphorylation of multiple downstream effectors that affect cytoskeletal structure, including myosin light-chain kinase (MLCK), paxillin, filamin A, cortactin, the PIX/COOL guanine nucleotide exchange factors, the LIM-kinases (LIMKs), Arpc1b, stathmin and tubulin cofactor B, many of which are upregulated in human tumors (Kumar *et al*., 2006). Therefore, unraveling the molecular mechanisms responsible for the temporal and spatial regulation of PAK-interacting proteins is important for a better

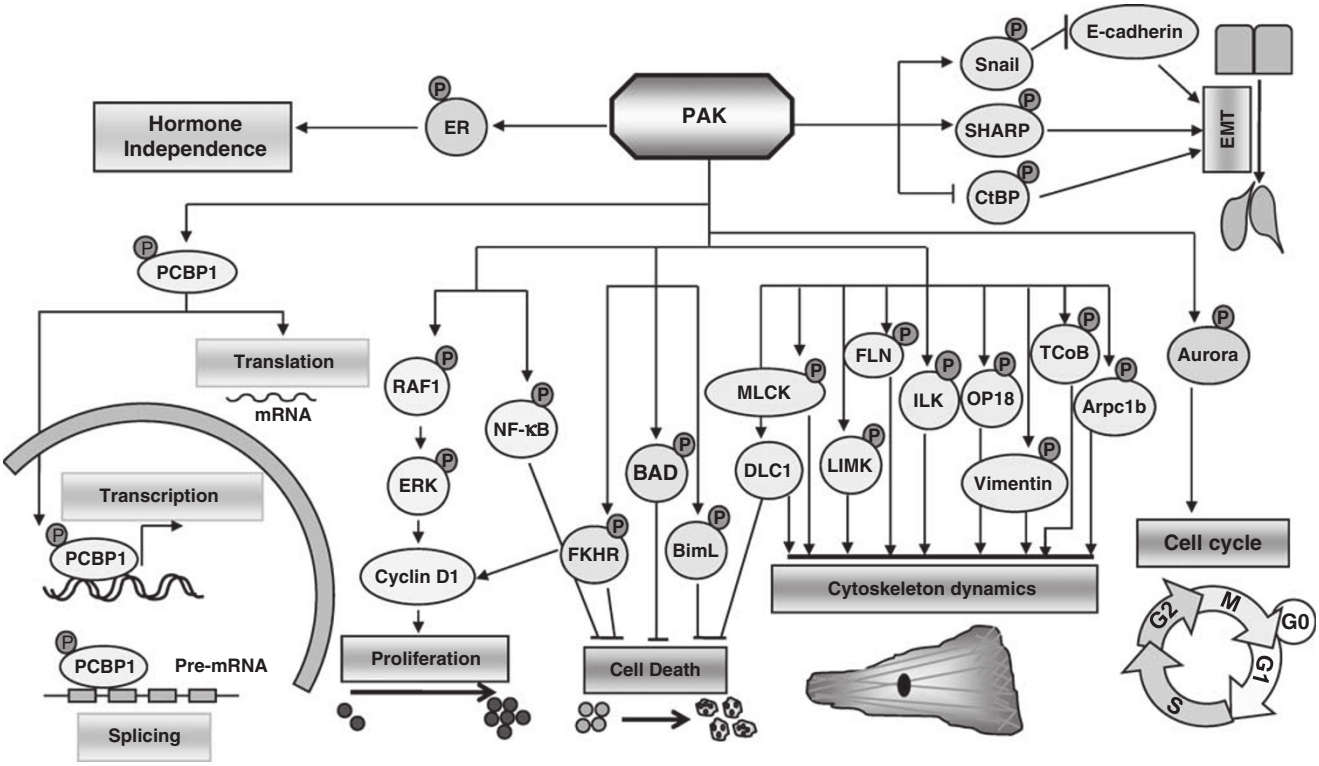

Figure 3 p21-activated kinase (PAK) stimulation of signaling cascades. PAKs activated by extracellular signals participate in various signaling networks. PAK activate the mitogen-activated protein kinase (MAPK) pathway by phosphorylating Raf1 in addition to nuclear factor-κB (NF-κB). PAKs also phosphorylate a number of regulators of the cytoskeleton such as myosin light-chain kinase (MLCK), LIM domain kinase (LIMK), filamin A, integrin-linked kinase (ILK), merlin, Arpc1b, tubulin cofactor B (TCoB) and stathmin (OP18). In addition, PAKs regulate survival and apoptotic pathways through phosphorylation of its effectors such as dynein light chain 1 (DLC1), BimL and the pro-apoptotic protein BAD. On translocation to the nucleus, PAK directly affects gene transcription. Several transcription factors and transcriptional coregulators such as FKHR, SHARP, C-terminal-binding protein 1 (CtBP1) and Snail homologue 1 (SNAI1) are substrates to PAK1. PAK1 coordinates transcription, splicing and translation by phosphorylating poly C-binding protein 1 (PCBP1). PAKs also regulate cell-cycle progression through phosphorylation of histone H3, Aurora A and polo-like kinase 1(PlK1).

understanding of the mechanistic contribution of PAKs in cellular transformation.

In mammalian cells, nonmuscle myosins are regulated by phosphorylation of their light chain (MLC) on Ser-19 by MLCK. The Rho target p160 Rho kinase phosphorylates and inhibits the MLC phosphatase activity, leading to the accumulation of phosphorylated MLC and, thereby, the increased contractility necessary for actin stress-fiber formation and cell spreading (Totsukawa *et al*., 2004). Activated PAK phosphorylates MLCK and inhibits its activity toward MLC, leading to reduced stress fibers (Chew *et al*., 1998). However, the effect of PAK on myosin activity appears to be cell-type specific.

PAK also regulates cell motility through regulation of focal adhesion dynamics. Paxillin, a multidomain protein, localizes to focal adhesions and forms a structural link between the ECM and the actin cytoskeleton and serves as an important site for signal transduction. In fact, paxillin functions as a scaffold protein, providing multiple docking sites for an array of structural proteins and signaling kinases such as FAK and Src in the focal adhesion. Phosphorylation of residues in the N-terminus of paxillin by these kinases regulates recruitment of downstream effector molecules such as vinculin, ARF, GAP, PKL, the exchange factor PIX and PAK to the focal adhesion (Turner, 2000). Once recruited, PAK phosphorylates paxillin on Ser-273, resulting in increased paxillin-GIT1 binding and localization of a GIT1/PIX/PAK signaling module near the leading edge, which culminates in a dramatic increase in migration, protrusion and adhesion turnover (Hoefen and Berk, 2006). PAK1 regulates actin dynamics at the leading edge of the motile cells by phosphorylating yet another substrate, filamin A. Interestingly, filamin A, in turn, stimulates PAK1 activity, leading to local activation of PAK (Vadlamudi *et al*., 2002).

Both PAK1 and PAK2 associate with another downstream effector, LIMK, which is overexpressed in breast and prostate cancer (Davila *et al*., 2003; Yoshioka *et al*., 2003; Bagheri-Yarmand *et al*., 2006). PAK activates LIMK by phosphorylating it at Thr-508, a residue within its activation loop. Activated LIMK phosphorylates cofilin, the actin-binding protein, and inactivates its F-actin-depolymerizing activity, thus modulating actin dynamics and cell motility (Edwards *et al*., 1999; Misra *et al*., 2005).

Following the loss of contractile constraints, the next phase in cancer cell metastasis involves degradation of the ECM. Actin structures are connected to the ECM by focal adhesions or podosomes. Typically, cells expressing focal adhesions display lower rates of motility, whereas invasive cells show the formation of dynamic F-actin-rich adhesion structures called podosomes. The speed of podosome assembly and disassembly generates high rates of motility, and the release of matrix metalloproteinases from podosomes facilitates degradation of the ECM (Block *et al*., 2008). Both PAK1 and PAK2 regulate migration and invasion by inhibiting the formation of podosomes by phosphorylation of caldesmon, one of the key regulators of actin dynamics that localizes to the podosomes (Morita *et al*., 2007). Actin dynamics also depend on Arp2/3 protein complex, an actin filament nucleating and organizing regulator. PAK1 can phosphorylate the Arpc1b subunit of this complex, which stimulates Arp2/3 complex assembly and regulates the directional motility of breast cancer cells (Vadlamudi *et al*., 2004b). Cortactin, an F-actin-binding protein, is enriched in dynamic cytoskeletal organelles such as podosomes, membrane ruffles and lamellipodia and is a substrate for PAKs (Webb *et al*., 2006). Furthermore, the *cortactin* gene is often amplified in breast cancer and in head and neck squamous cell carcinoma and is associated with lymph node metastasis and poor prognosis (Buday and Downward, 2007). PAK-mediated phosphorylation of cortactin reduces its binding affinity for F-actin and for Arp2/3 and affects the stability of branched actin filaments (Lua and Low, 2005).

Migration involves dynamic changes not just in the actin cytoskeleton but also in the microtubule network. Stathmin, also called oncoprotein 18 (OP18), is a microtubule-destabilizing protein that is overexpressed in sarcomas and contributes to local tumor invasiveness (Cassimeris, 2002; Belletti *et al*., 2008). PAK1 phosphorylation of stathmin inactivates it, which results in the stabilization of microtubules at the leading edge of motile cells (Wittmann *et al*., 2004). PAK1 also regulates microtubule dynamics by phosphorylating tubulin cofactor B, which contributes to α- and β-tubulin heterodimer assembly and is often upregulated in human breast tumors (Vadlamudi *et al*., 2005). In recent years, yet another PAK effector, guanylyl cyclase, has been shown to regulate cell motility. Guanylyl cyclases catalyse the conversion of GTP to the second messenger, cyclic GMP, which has also been implicated in the regulation of cell motility. Although PAK activity is required to promote guanylyl cyclase activity, guanylyl cyclases do not appear to be phosphorylated by activated PAK. In fact, it appears to be an indirect event, wherein activated Rac promotes a conformational change in PAK that enables it to bind to guanylyl cyclases and, presumably, promote a subsequent conformational change in the guanylyl cyclase that leads to its activation (Settleman, 2007). Thus, PAKs act as signaling nodules that link upstream stimuli to a very complex network of signal transduction pathways, resulting in cytoskeletal remodeling.

Cytoskeleton-regulated gene expression

In addition to its functions in cytoskeletal remodeling, cell motility and invasion, PAK also influences cancer cell biology through its nuclear functions. Tumor progression involves the activation and repression of various genes that are involved in essential cellular processes. It is not clear whether PAKs participate in gene expression in the nucleus, but nuclear translocation of PAK in response to stimulus and a direct association between PAK1, presumably as a part

of the PAK-multiprotein complex, and specific gene chromatin and enhancer elements partly explain PAK-dependent gene transcription and translation (Singh *et al*., 2005).

Several transcription factors and transcriptional coregulators have been identified as PAK1-interacting substrates, including the forkhead transcription factor (FKHR), estrogen receptor-α (ER-α), SHARP, C-terminal-binding protein 1 (CtBP1) and Snail homologue 1 (SNAI1). Of these, both CtBP1 and Snail are important in the process of epithelial-mesenchymal transition (EMT) (Grooteclaes and Frisch, 2000; Come *et al*., 2004). Although EMT is important in many developmental processes, such as gastrulation and neural crest migration, its deregulation can lead to tumor progression, and EMT is often seen in cells with PAK overexpression or PAK hyperactivation (Yang *et al*., 2005). PAK1 also phosphorylates ER at Ser-305 and promotes its transactivation functions, leading to increased cyclin D1 expression and conferring growth advantage and hormone independence to breast cancer cells (Nheu *et al*., 2004; Rayala *et al*., 2006). PAK1-mediated phosphorylation of PCBP1 on Thr-60 and Thr-127 also stimulates transactivation of the initiation factor (eIF4E) gene promoter and pre-mRNA splicing (Meng *et al*., 2007). In addition to its function in the regulation of transcription, PAK1 also regulates translation. Activation of PAK2 leads to the binding and phosphorylation of eIF4G, which inhibits the association of eIF4E with m(7)GTP, reducing translation efficiency (Ling *et al*., 2005).

PAK signaling and cell-cycle progression

The cell cycle is a tightly regulated process that involves a coordinated orchestra of multiple regulators, and disruption of such regulatory steps leads to uncontrolled cell-cycle progression. A large body of evidence indicates that phosphorylation has a pivotal function in the cell-cycle checkpoints, and therefore, it is not surprising that kinases such as cyclin-dependent kinases, polo-like kinase 1 (Plk1), Aurora kinases and PAKs might contribute to the process of tumorigenesis by affecting cell-cycle progression (Schatten, 2008).

PAK1 is phosphorylated at Thr-212 by cyclin B1/Cdc2 during mitosis in mammalian fibroblasts (Banerjee *et al*., 2002; Thiel *et al*., 2002). This phosphorylation is unique to PAK1 because Thr-212 is not conserved in PAK2 or PAK3. The mitotic phosphorylation of PAK1 does not alter the ability of the small GTPase to stimulate the PAK kinase, but it alters its association with the binding partners that have a function in morphological changes associated with cell division. PAK1 also colocalizes with histone H3 in condensing chromatin and phosphorylates it on Ser-10, which is required for transcription activation as well as for the onset of mitosis (Li *et al*., 2002). In mitotic cells, phosphorylated PAK1 is preferentially localized on centrosomes and spindles, prompting a long period of speculation that PAK1 might be important in centrosome dynamics. Zhao *et al*. (2005) showed that the PIX/GIT1 complex (but not Cdc42) is involved in localizing PAK to the centrosome, where it undergoes activation. Once activated, PAK dissociates from the PIX/GIT1 complex and binds to and phosphorylates Aurora kinase A on Thr-288 and Ser-342, the key sites for Aurora kinase activation and Plk1 on Ser-49 in mitosis (Zhao *et al*., 2005; Maroto *et al*., 2008). As mitosis progresses, PAK1 localizes to the spindle midbody and finally to the contractile ring during cytokinesis (Li *et al*., 2002). Thus, PAK might participate in all phases of the cell cycle.

Cytoskeleton-modulated cell proliferation or apoptosis

In the normal cell, DNA is duplicated during mitosis and distributed equally between the two daughter cells. Cancer cells, however, owing to the overexpression and hyperactivation of mitotic kinases, such as PAK, often display aneuploidy due to improper segregation of chromosomes (Vadlamudi *et al*., 2000). Typically, cells with mitotic catastrophe undergo apoptosis and are eliminated; however, some cells develop tolerance to become polyploidy and continue to proliferate and give rise to cancerous phenotypes. Depending on the cellular context, PAKs have been shown to either promote cell growth or push cells to apoptosis. Multiple mechanisms exist through which PAK1 promotes cell survival. In the presence of a cell survival signal, PAK1 phosphorylates Bad on Ser-112 and Ser-136, inhibits its interaction with the Bcl2 family members and exerts anti-apoptotic actions (Schurmann *et al*., 2000). In addition, PAK1 interacts with dynein light chain 1 (DLC1), which typically sequesters BimL and prevents it from inactivating the survival functions of Bcl2 (Vadlamudi *et al*., 2004a). However, under apoptotic stimuli, DLC1–BimL dimers are released from the dynein motor complex, which frees BimL to inhibit Bcl2 (Puthalakath *et al*., 1999; Bouillet *et al*., 2002). PAK1 phosphorylates DLC1 and BimL and triggers their degradation, thus blocking the pro-apoptotic signal of BimL (Vadlamudi *et al*., 2004a). PAK1 also inhibits apoptosis by phosphorylating and inactivating FKHR. Interestingly, FKHR phosphorylation is accompanied by its cytoplasmic accumulation and its inability to activate pro-apoptotic target genes (Mazumdar and Kumar, 2003). PAK1-mediated activation of cellular pathways also leads to an enhanced cell survival.

Unlike PAK1, PAK2 (also known as γ-PAK) has a dual function and regulates both cell survival and cell death pathways, depending on the milieu. PAK2 is activated through at least two signaling pathways: one requires phosphoinositide 3-kinase and/or AKT activity, whereas the other requires caspase activity (Walter *et al*., 1998; Roig and Traugh, 1999). Cellular stresses such as DNA damage, hyperosmolarity and serum starvation activate the PAK2 enzyme to generate a proteolytic fragment, the PAK-2p34 (Roig and Traugh, 1999; Ling *et al*., 2005). Binding of Cdc42 to

full-length PAK2 translocates PAK2 to the endoplasmic reticulum, where it is autophosphorylated and activated. Activation of full-length PAK2 promotes cell survival by phosphorylating Bad and reducing the interaction between Bad and Bcl-2 or Bcl-x(L), which increases the association between Bad and 14-3-3tau, leading to cell survival (Jakobi *et al*., 2001). In contrast, proteolytic activation of PAK-2p34 leads to apoptosis (Jakobi *et al*., 2003). In addition, this pathway is also negatively regulated by PS-GAP, a GTPase-activating protein (GAP) that inhibits apoptosis by interacting with and suppressing PAK-2p34-associated kinase activity (Koeppel *et al*., 2004).

The third member of the group I PAKs, PAK3, is primarily expressed in the brain. The *PAK3* gene encodes four isoforms, PAK3a, PAK3b, PAK3c and PAK3cb of 544, 559, 622 and 667 amino acids, respectively. PAK3c and PAK3cb are newly identified isoforms that include insertion of exons in the PBD/KID, which strongly increase the kinase activity and modify the GTPase binding to these isoforms (Kreis *et al*., 2008). PAK3b isoform has an insertion in the PBD/KID sequence, but it is outside the GTPase Cdc42- and Rac-binding domain, allowing the kinase to be active in the absence of GTPase binding. PAK3a is activated by both Rac and Cdc42 GTPases. Although Rac is a weak activator of PAK3a, it is important in recruiting PAK3a to the membrane. In contrast, Cdc42 recruits and activates PAK3a but only recruits PAK3b to the membrane because it is already active (Rousseau *et al*., 2003).

Similar to the other two group I PAK members, PAK3 has a function in the regulation of cytoskeleton dynamics. Of the many focal adhesion proteins, only paxillin-α and paxillin-β isoforms interact with PAK3 and link both the kinase-inactive and kinase-active forms of PAK3 to integrins (Hashimoto *et al*., 2001). Another effector of PAK3 is Raf-1. PAK3 phosphorylates Raf-1 on Ser-338, leading to Raf activation independent of Ras GTPase (King *et al*., 1998). PAK3 also confers a survival advantage to cancer cells by activating NADPH oxidase (an enzyme that regulates the intracellular source of reactive oxygen species (ROS)) and altering the redox potential of the cells. The redox state is governed by the balance between ROS and the antioxidant levels in the cell. PAK1 has a crucial function in the regulation of both NADPH oxidase and metabolic pathways. PAK1 phosphorylates the p47 (phox) subunit (Knaus *et al*., 1995), whereas PAK3 phosphorylates the p67 (phox) subunit of NADPH oxidase (Ahmed *et al*., 1998), which leads to the activation of NADPH oxidase.

Interestingly, of all the PAKs, PAK3 is the only one known to be associated with a human genetic disease. Mutations in the *PAK3* gene are associated with X-linked, nonsyndromic mental retardation (MRX) syndromes. Three types of mutations have been reported—MRX30, R67C and A365E. MRX30 mutation generates a truncated, kinase-dead mutant. R67C mutation is expected to affect GTPase binding, whereas the A365E mutation affects a highly conserved region within the protein kinase domain (Bienvenu *et al*., 2000; Gedeon *et al*., 2003).

Group II PAKs

Group II PAKs are structurally distinct from group I PAKs (Figure 2). They contain an N-terminal PBD and a C-terminal kinase domain, but lack other motifs found in group I PAKs. The degree of similarity between the PBD and kinase domains in the group II PAKs is much less than that in the group I PAKs. PAK4-6 bind to activated Cdc42 and, to a lesser extent, to Rac, but the activity of these kinases is not appreciably enhanced upon binding to the GTPases. In fact, interaction with Cdc42 induces the translocation of group II PAKs to different cellular compartments. Binding to Cdc42 results in PAK4 translocation to the Golgi apparatus (Abo *et al*., 1998; Callow *et al*., 2002). PAK5 localizes to mitochondria and the nucleus but in a Cdc42-independent manner (Cotteret *et al*., 2003; Cotteret and Chernoff, 2006; Wu and Frost, 2006). Thus, unlike with the group I PAKs, the interaction of group II PAKs with GTPases has no influence on kinase activity. However, similar to the group I PAKs, group II PAKs are important in cell motility and survival.

PAK4, the first identified member of group II PAK, is a target for Cdc42 and undergoes autophosphorylation on Ser-474 (Abo *et al*., 1998). In cellular studies, PAK4 has a function in oncogenic transformation with PAK4 activity required for Ras-driven, anchorage-independent growth (Callow *et al*., 2002). Expression of active PAK4 (S474E) mutant has transforming potential, leading to anchorage-independent growth of NIH3T3 cells. A kinase-inactive PAK4 (K350A, K351A) efficiently blocks transformation by activated Ras and inhibits anchorage-independent growth of HCT116 colon cancer cells. Expression of activated PAK4 in fibroblasts leads to a transient induction of filopodia, dissolution of stress fibers and loss of focal adhesions (Qu *et al*., 2001). The reorganization of the actin cytoskeleton is dependent on PAK4 kinase activity and on its interaction with Cdc42. PAK4 also regulates cytoskeletal changes through the modulation of LIMK1's activity. Activated PAK4 phosphorylates LIMK1 and stimulates its ability to phosphorylate cofilin (Dan *et al*., 2001). Interestingly, fibroblasts expressing activated PAK4 show oncogenic transformation (Cammarano *et al*., 2005). In primary fibroblasts, activated PAK4 inhibits cell proliferation and promotes premature senescence. Activated PAK4 also protects cells against apoptotic cell death by phosphorylating the pro-apoptotic protein Bad and by inhibiting caspase activation (Gnesutta *et al*., 2001; Gnesutta and Minden, 2003). PAK4 mediates morphological changes through its association with the Rho-family guanine nucleotide exchange factor GEF-H1 (Callow *et al*., 2005). GEF-H1 is involved in microtubule dynamics and when mutated causes oncogenic transformation (Krendel *et al*., 2002; Brecht *et al*., 2005). In humans, PAK4 is expressed at low levels in normal tissue, but its expression is upregulated in a large variety of human tumor cell lines

(Callow *et al*., 2002) and Ras-dependent human tumors (Parsons *et al*., 2005; Chen *et al*., 2008; Kimmelman *et al*., 2008).

PAK5 (also known as PAK7) is highly expressed in the brain and contains an N-terminal CRIB motif and a C-terminal kinase domain but lacks a PAK inhibitory domain. PAK5 also contains an autoinhibitory fragment similar to that of PAK1, which is absent from the other group II PAK family members (Pandey *et al*., 2002). PAK5 is structurally related to PAK4 and PAK6 within its CRIB domain. PAK5 binds to the GTPases Cdc42 and Rac, but these GTPases do not regulate PAK5 kinase activity. PAK5 is constitutively active, but its autophosphorylation can be further stimulated by the GTP-bound form of Cdc42. PAK5 operates downstream of Cdc42 and Rac and antagonizes Rho in the pathway, leading to filopodia formation and neurite outgrowth (Dan *et al*., 2002). PAK5 also stabilizes microtubules by negatively regulating MARK2, a kinase that promotes microtubule disruption by phosphorylating microtubule-associated proteins such as tau (Timm *et al*., 2006). PAK5 has different effectors depending on its localization. In the cytosol, PAK5 activates the JNK kinase pathway, and in the mitochondria it has a function in survival signals (Dan *et al*., 2002; Cotteret and Chernoff, 2006). PAK5 protects cells from apoptosis by phosphorylating Bad on Ser-112 and preventing its localization to mitochondria (Cotteret *et al*., 2003). Although PAK5 itself is constitutively localized to mitochondria, this localization is independent of kinase activity or Cdc42 binding. The function of PAK5 in cancer was not known until a recent study showed that point-mutated PAK5 contributed to human cancers (Greenman *et al*., 2007).

On the basis of its homology to the PAK family, PAK6 was originally cloned from prostate cancer cells as an androgen receptor (AR)-interacting protein (Yang *et al*., 2001). PAK6 is overexpressed in both primary and metastatic prostate cancer cells and contributes to prostate cancer development and progression after androgen deprivation therapy (Kaur *et al*., 2008). PAK6 showed very high, although restricted expression pattern with PAK6 detected in brain, testis, prostate and placenta (Callow *et al*., 2002). In most of the tumor cell lines, PAK6 was expressed at low levels or was not detectable. For example, two of the eight colon cancer cell lines exhibited high PAK6 mRNA levels, whereas PAK6 expression could not be detected in normal colon tissue (Callow *et al*., 2005). Like PAK4, PAK6 possesses constitutive basal kinase activity, and its activity is not modulated by binding to active Rac or Cdc42 but is stimulated by binding to AR. PAK6 interacts not only with AR but also with ER-α. PAK6 interaction with ER-α leads to the repression of ER-α transcriptional activity (Lee *et al*., 2002). PAK6 is primarily localized in the cytoplasm, but in the presence of ligand AR, PAK6 translocates to the nucleus. Moreover, the binding of activated PAK6 to AR inhibits AR from binding to transcriptional coactivators, as well as inhibiting its transactivation function (Yang *et al*., 2001); thus, PAK6 is important in steroid-hormone-mediated signal transduction.

Lessons from PAK-null mice

Although data from tissue culture model systems have provided deep insights into the complex regulation and functions of the PAK signaling pathways, we have just begun to utilize PAK-null mice to study the function of PAK *in vivo* (Figure 4). PAK1 knockout mice are viable, healthy and fertile but have a defective immune response. In contrast, PAK2 knockout mice are

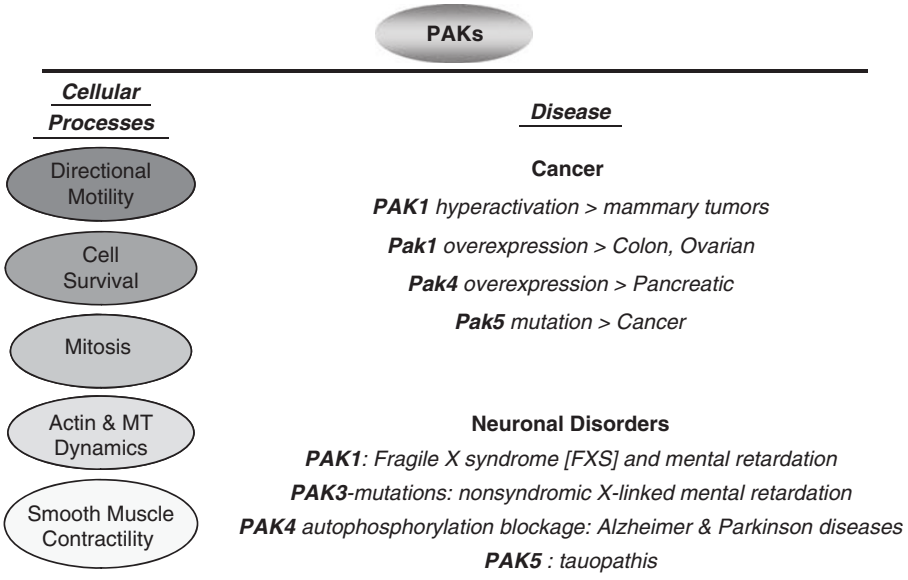

Figure 4 Altered expression of the p21-activated kinase (PAK) family members. Cell proliferation, differentiation and migration are tightly regulated processes, and perturbation of this balance is associated with tumorigenesis. A function for PAKs has been established both in controlling normal cellular processes and in cancer progression. PAKs are either overexpressed or hyperactivated in various cancers and neurological disorders.

embryonically lethal due to multiple developmental abnormalities (Arias-Romero and Chernoff, 2008). Because PAK3 expression is brain specific, it is not surprising that PAK3 knockout mice show impaired synaptic plasticity and deficiencies in learning and memory (Meng *et al*., 2005). Similar to PAK2 knockout, the PAK4 knockout model is embryonically lethal, but these mice probably die of a heart defect. The embryos from PAK4 knockout mice also show neuronal abnormalities such as defective neuronal differentiation and migration of motor neurons of the developing spinal cord and neural tube. In addition, the neural tube in these mice is improperly folded (Qu *et al*., 2003). The phenotype of the PAK5-null mice is completely different from that of PAK4-null mice. Owing to the function of PAK5 in neurite outgrowth in neuroblastoma cells, one would expect some degree of neuronal defects in PAK5-null mice, but the mice develop normally and are fertile (Li and Minden, 2003). To date, there has been no published report of a PAK6 knockout mouse model. The expression patterns of PAK5 and PAK6 are very similar, and PAK6 mRNA levels are significantly higher than those of PAK4 in the adult mouse brain, indicating that these kinases can have overlapping functions and can compensate for the absence of one another. In brief, among the PAK family, only PAK2 and PAK4 are essential for development.

Negative regulation of the PAK pathway

Although we have learned a lot about the positive regulation and functions of PAKs, our understanding of the endogenous negative regulators of PAKs continues to lag behind. Two closely related human protein phosphatases (PPs), POPX1 and POPX2, efficiently dephosphorylate PAK1. POPX1 and POPX2 bind to various forms of PIX- and PAK1-containing complexes, allowing PAK to cycle rapidly between active and inactive states (Koh *et al*., 2002). Similarly, PAK3 is identified as a substrate for PP1 and PP2A and an Mg^{2+}/Mn^{2+}-dependent phosphatase(s). PP1α and PP2A dephosphorylate Thr-421 in the activation loop of PAK3, whereas PP1α, PP2A, PP2B and PP2C dephosphorylate PAK3 at Ser-139 (Zhan *et al*., 2003). One newly identified mechanism is a negative-inhibitory loop generated by Cdc42 homologous protein (Chp). Chp inhibits PAK1 functions through ubiquitination and proteasome-mediated degradation. Chp-induced degradation of PAK depends on its PBD and kinase activity and on the autophosphorylation of PAK1, but not on the PIX- and Nck-binding sites (Weisz *et al*., 2007). Another PAK-interacting protein, hPIP1, negatively regulates PAK kinase activity by blocking autoactivation and/or interactions with GTPases. hPIP1 contains G-protein β-like WD repeats and shares sequence homology with Skb15 (the fission yeast PAK regulator) and MAK11 (the budding yeast protein) (Xiao *et al*., 2002). Similarly, the neurofibromatosis type 2 protein merlin interacts with the N-terminal regulatory domain of PAK1 and inhibits Cdc42/Rac1-stimulated kinase activity (Xiao *et al*., 2002). The Cdc2-related kinase PITSLRE and the $\alpha5\beta1$-integrin binding partner Nischarin also inhibit the ability of PAK1 to phosphorylate substrates (Chen *et al*., 2003; Alahari *et al*., 2004).

Therapeutic challenges

Due to the signal-dependent activation of group I PAKs, designing specific inhibitors to interfere with upstream regulators of PAK has been somewhat successful. Several small molecule inhibitors such as CEP-1347, SRC and ETK tyrosine kinase inhibitors, AG 879 and FK228 have been shown to effectively inhibit PAK activity in a variety of experimental systems (He *et al*., 2004; Nheu *et al*., 2004; Hirokawa *et al*., 2005). More recently, PAK1 activation has been also targeted by allosteric inhibitor such as IPA-3 (Deacon *et al*., 2008). However, it remains to be seen whether these PAK inhibitors are also effective and exhibit desired target selectivity in physiological whole-animal setting. Given the diversity and overlapping nature of PAK regulators and effectors, it is likely that some of these inhibitors will have to overcome the expected problem of specificity and redundancy before these agents could move to clinical development. Targeting group II PAKs is a bigger challenge because they do not require external stimuli for activation and are constitutively active. Although our present understanding of PAK signaling points to interesting possibilities for PAK therapy, further studies are required to fully elucidate PAK functions in normal and cancer cells. It is expected that the development of inhibitors to this family of enzymes will further unravel the function of PAKs in tumorigenesis and help to establish the PAK family as therapeutic targets.

Conflict of interest

The authors declare no conflict of interest.

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